

## STUDIES ON THE ADSORPTION OF DIPHTHERIA TOXOID BY ALUMINUM OXIDE HYDRATE GELS

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During studies on the properties of colloidal aluminum hydroxide gels (1, 2, 3, 4, 5), we became interested in their adsorptive properties, specially of those related to the adsorption of viruses. In these studies, it is important to know the adsorptive power of the gels in aqueous media: the measurements of the amount of adsorption of dyes (6, 7) and of diphtheria toxoid (8) are the only indirect methods for evaluating the adsorptive power for viruses of aluminum hydrate gels.

From previous experiments (7) on the adsorption of diphtheria toxoid by aluminum hydroxide gels of different crystalline structures and morphologies, there was evidence that the amount of adsorption is greatly influenced by the nature of the anions present in the media. This observation is by no means new, since the effect of phosphate buffer on the elution of diphtheria toxoid adsorbed on aluminum hydroxide gel was described by Schmidt and Oerskov in 1935 (9). The same effect on other proteins and viruses has already been discussed by Sheppard in studies on the chromatographic adsorption of serum proteins and bacteriophages on silicic acid and aluminum oxide gels (10), by McLaren on the adsorption of enzymes on kaolinite (11), and by Holt (12) and Mason (13) on the adsorption of diphtheria toxoid by aluminum hydroxide and phosphate gels. However, no systematic studies are known on the influence of the nature and charge of the anions present in the medium containing the toxoid on the adsorptive power of the adsorbent. It is the aim of this paper to present the results of a study on the effect of some anions upon the adsorptive power of different aluminum oxide hydrate gels.

### MATERIAL AND METHODS

*Diphtheria Toxoid* — Prepared according to the standard methods of the New York State Department of Health (14). The concentration of the toxoid was obtained by isoe-

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electric precipitation with HCl at pH 3.2, separation of the precipitated toxoid by centrifugation and dissolution in the minimum amount of M/15 phosphate ( $\text{KH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4$ ) buffer of pH 7.2. This stock solution was diluted to the desired titer of toxoid, with the same phosphate buffer or with 0.85% NaCl solution. The toxoid samples for the study of the influence of anions of different valency were prepared in the same way, except that the washed toxoid was dissolved in glycine-potassium acetate buffer of pH 7.2 (7, 15). The toxoid solutions were kept in an ice box; different batches of diphtheria toxoid were used in the experiments. The titer of the toxoids was determined by the Ramon toxin and antitoxin method (16) before each experiment, the results being given in Lf units. Since M/15 phosphate buffer is used in the standard methods for preparation and dilution of the toxoid solutions (14), the same concentration of the salts of the other anions for investigating the influence of the anion was used.

*Aluminum Hydroxide Gels.* — The following aluminum hydroxide gels were used (7) in the adsorption experiments: 1) C-gamma gel according to Willstaetter, prepared by slow aging of C-alpha gel; the sample was aged more than two years; X-ray diffraction showed it to consist of the mixture of Gibbsite ( $\alpha\text{-Al}_2\text{O}_3 \cdot 3\text{H}_2\text{O}$ ) and of Bayerite ( $\gamma\text{-Al}_2\text{O}_3 \cdot 3\text{H}_2\text{O}$ ), with the predominance of the former structure, as reported in a previous paper (2); the sample contains small amounts of ammonium sulphate deriving from the preparation method. 2) Boehmite ( $\gamma\text{-Al}_2\text{O}_3 \cdot \text{H}_2\text{O}$ ) from the reaction of amalgamated aluminum with boiling conductivity water according to Fricke and Jockers; this sample consists mainly of irregular plates, as reported in a previous paper (1); it contains practically no electrolytes. 3) Bayerite from the reaction of amalgamated aluminum with conductivity water at room temperature, followed by aging of about two weeks, according to Fricke and Jockers; this sample consists of pure Bayerite, as triangular or hourglass-shaped somatoids (2). 4) Aluminum hydroxide gels of Boehmite structure prepared by reaction of aluminum chloride with sodium carbonate or ammonium hydroxide in the hot or at room temperature and purified by washing in the centrifuge or by dialysis against distilled water; Schmidt's gel was prepared from ammonium alum and ammonium hydroxide followed by washing and autoclaving at  $120^\circ\text{C}$ ; all these samples are composed of fibrils similar to those observed in C-beta gels as described in previous papers (1, 2, 3). 5) Amorphous aluminum hydroxide gels ( $\text{Al}(\text{OH})_3$ ), recently precipitated (16a); the aluminum hydroxide gel was also precipitated *in situ* by reaction of aluminum chloride with ammonium or potassium hydroxide in stoichiometric amounts, at room temperature; hence, the medium contained ammonium or potassium chloride from the reaction. The properties of these aluminum hydroxide gels are described in detail in the references (1, 2, 3, 4, 5 and 16a).

The amount of aluminum contained in the gels was determined with 8-hydroxyquinolein by a volumetric method (17). For comparison of the adsorptive power, the solid content of the gels used is given as the  $\text{Al}_2\text{O}_3$  content, in view of the different structural formulas and chemical compositions of the gels. The adsorption experiments were made by allowing the gels to be in contact with the toxoid for about one hour at room temperature, with shaking in intervals, followed by centrifugation; the titer of toxoid left in the supernatant was measured by the Ramon toxin and antitoxin method as described in a previous paper (7).

## RESULTS AND DISCUSSION

*Influence of the Phosphate Ion.* — It was first reported by Schmidt and Oerskov that phosphate ion prevents the adsorption of diphtheria toxoid (9).



Some qualitative experiments were conducted in order to study the behaviour of aluminum hydroxide gels of different crystalline structure and shape, using a fixed amount of toxoid in a medium containing M/15 phosphate buffer of pH 7.2. Typical experiments are shown in Table I.

TABLE I

INFLUENCE OF PHOSPHATE ION ON ADSORPTION OF DIPHTHERIA TOXOID BY WILLSTAETTER'S C-GAMMA GEL

Mg of $Al_2O_3$ contained in 20 ml of final mixture		Control	0.125	0.250	0.500	1.000	2.000	4.000	8.000
Experiment I	Toxoid: ml of toxoid diluted in M/15 phosphate buffer	10.0	10.0	10.	10.0	10.0	10.0	10.0	10.0
	Diluent: ml of M/15 phosphate buffer to dilute to 20 ml								
	Number of Lf units in 20 ml of mixture	18	18	18	18	18	18	18	18
	Number of Lf units adsorbed from 20 ml	0	0	0	0	0	0	0	0
Experiment II Identical amounts of reagents as in Exp. I, with the same toxoid, but using 0.85% NaCl solution as diluent instead of M/15 phosphate buffer	Number of Lf units adsorbed from 20 ml	0	0	0	0	0	0	0	0
Experiment III Identical amounts as in Exp. II, but using toxoid diluted with 0.85% NaCl solution instead of M/15 phosphate buffer and using 0.85% NaCl solution as diluent	Number of Lf units adsorbed from 20 ml	0	0	0	0	0	0	0	0

The final volume of 20 ml in each tube was completed with 0.8% NaCl solution or with M/15 phosphate buffer of pH 7.2. A constant amount of 18 Lf units of toxoid was added to each tube. The mixture was homogenized by careful shaking, left at room temperature for one hour and centrifuged at 1560 r.p.m. for one hour. After centrifugation, the supernatant was collected and the number of Lf's titrated as described. A control was used with an identical series of tubes containing distilled water in place of the aluminum oxide hydrate gel. Titrations of the supernatants and of controls were made at the same time.



In experiment I of this table, only phosphate ion existed in the medium, with the possible exception of a small amount of sulphate ion from C-gamma gel: no adsorption occurred. In experiment II, the final volume was completed with 0.85% NaCl solution instead of phosphate buffer; the medium contained phosphate ion in a constant concentration from the toxoid and a varying amount of chloride ion: no adsorption occurred. In experiment III, the toxoid was dissolved in the smallest amount possible of phosphate buffer, but diluted with 0.85% NaCl solution in order to obtain 370 Lf units in 10 ml; in the adsorption experiment the final volume was also completed with this solution; the medium had a small constant amount of phosphate ion and an increasing concentration of chloride ion. A significant amount of adsorption of 3 Lf units was observed in the tubes containing 4 and 8 mg of  $\text{Al}_2\text{O}_3$ . These experiments confirm the fact reported (7, 9) that phosphate ion has a strong inhibiting power on the adsorption of diphtheria toxoid by Willstaetter's C-gamma gel, and show furthermore that the inhibiting power depends on the amount of phosphate present in the medium.

Similar experiments were conducted in order to investigate whether this inhibiting power is significantly influenced by the crystalline structure of the aluminum hydroxide gels (7). These experiments are summarized in Table II, and the amounts of reagents are the same as those listed in Table I.

TABLE II

COMPARISON OF THE INFLUENCE OF PHOSPHATE ION ON THE ADSORPTION OF DIPHTHERIA TOXOID BY ALUMINUM HYDROXIDE GELS OF DIFFERENT CRYSTALLINE STRUCTURE

Aluminum hydroxide gel in 20 ml of mixture	mg of $\text{Al}_2\text{O}_3$	0	0.125	0.25	0.50	1.00	2.00	4.00	8.00
Bayerite (from amalgamated aluminum)	Exp. I	0	0	0	0	0	0	0	0
	Exp. II	0	0	0	0	0	0	0	0
	Exp. III	0	0	0	0	0	0	0	0
C-gamma gel of Willstaetter (Bayerite + Gibbsite)	Exp. I	0	0	0	0	0	0	0	0
	Exp. II	0	0	0	0	0	0	0	0
	Exp. III	0	0	0	0	0	0	0	0
"New b" Willstaetter ( $\text{AlCl}_3 + 4\text{OH}$ + dialysis - Bayerite + Gibbsite)	Exp. I	0	0	0	0	0	0	0	0
	Exp. III	0	0	0	0	0	3	3	3
Boehmite (from amalgamated aluminum)	Exp. I	0	0	0	0	0	0	0	6
	Exp. II	0	0	0	0	0	0	6	8
	Exp. III	0	0	3	3	8	18	18	18
Boehmite ( $\text{AlCl}_3\text{NH}_4\text{OH}$ pp. in the boiling temperature)	Exp. I	0	0	0	0	0	0	0	0
	Exp. III	0	3	3	3	6	10	12	14
Boehmite ( $\text{AlCl}_3\text{Na}_2\text{CO}_3$ pp. at room temperature)	Exp. I	0	0	0	0	0	0	0	0
	Exp. III	0	0	0	0	0	0	0	0
Boehmite ( $\text{AlCl}_3 + \text{NH}_4\text{OH}$ pp. at room temperature)	Exp. I	0	0	0	0	0	3	3	3
	Exp. III	0	3	3	6	8	14	18	18

NOTE: the numbers in the table are the number of Lf units absorbed from 20 ml of suspension.



The total volume of mixture was also 20 ml; the total number of Lf units before adsorption was 18. In experiment I, the toxoid was dissolved and diluted in phosphate buffer; the final volume was completed with the same buffer. In experiment II, the toxoid was dissolved and diluted in phosphate buffer; the final volume was completed with 0.85% NaCl solution. In experiment III, the toxoid was dissolved in phosphate buffer, but diluted in 0.85% NaCl solution; the final volume was also completed with 0.85% NaCl solution. Consequently, the phosphate ion content of the medium is higher in experiment II than in experiment III. The numbers listed in the table represent the number of Lf units adsorbed by the gel from the 18 Lf units existing in the 20 ml of suspension of toxoid plus gel. The order in the table follows roughly the decreasing particle size or increasing surface area as evaluated from electron microscopy and from the adsorption of alizarin by the gels (1, 2, 3, 4, 5, 7, 16a); Bayerite gel from amalgamated aluminum has the smallest surface area.

These experiments demonstrate that phosphate ion has an inhibiting power on the adsorption of diphtheria toxoid by aluminum hydroxide gels of different crystalline structures and surface areas. This inhibiting power depends on the concentration of phosphate ion present and decreases, if the concentration of phosphate is decreased; with the exception of Boehmite gel from the reaction of amalgamated aluminum with boiling water, M/15 phosphate buffer, in the concentration and volume used, is enough to prevent adsorption of toxoid on the amounts of aluminum hydroxide gels of various crystalline structures and particle sizes listed in Table II; chloride ion does not inhibit adsorption. In the conditions used in the experiments, Boehmite gels, from amalgamated aluminum and from  $\text{AlCl}_3 + \text{NH}_4\text{OH}$  precipitated at room temperature, have a greater adsorptive power for toxoid than Bayerite from amalgamated aluminum. The explanation for these differences may probably be found rather in the difference of the particle size and/or surface area than in the crystalline structure (7).

Since Holt and Mason's experiments (12, 13) showed that aluminum phosphate adsorbs diphtheria toxoid and that phosphate ion also influences this adsorption, it was decided to investigate in detail the nature of this inhibiting power of phosphate ion; since its mechanism is unknown (7), the first working hypothesis which was investigated to explain this difference is that the inhibiting power is due to a difference in valency or charge of the anion and that the inhibition of the adsorption occurs by a mechanism similar to that in the coagulation of liophobic colloids by adsorption of counter ions in the diffuse double layer around the particles of the positive aluminum hydroxide gels, as discussed by Weiser (18).



*Influence of the Anion Charge on the Adsorption of Toxoid.* — A series of experiments was performed on the adsorption of diphtheria toxoid, using toxoid dissolved in M/15 glycine-acetate buffer of pH 7.2 (7), instead of phosphate, in order to have a monovalent anion in the medium; it was admitted that the glycine and acetate ions would behave like the ion of chloride, which had not inhibited the adsorption of toxoid, and this hypothesis was confirmed by experiments similar to that described in Table III. These results are in disagreement with Holt's (12), who found that amino acids from casein hydrolysate inhibit the adsorption of toxoid by aluminum hydroxide gel. With this toxoid solution, it was possible to investigate the influence of the charge of ions like chloride, sulphate, phosphate or citrate and ferrocyanide on the adsorption of diphtheria toxoid; potassium triphosphate was used in place of diphosphate in order to have a trivalent phosphate ion. Potassium salts were used in all experiments in view of the availability of all salts of the desired anions. A protocol of a typical adsorption experiment is summarized in Table III.

TABLE III

TYPICAL PROTOCOL OF AN ADSORPTION EXPERIMENT WITH POTASSIUM SALTS OF DIFFERENTLY CHARGED ANIONS

TUBES	I	II	III	IV	V	VI	VII	VIII
Diphtheria toxoid Nr. 55.44 in ml (400 Lf/ml)	0.250	0.383	0.588	0.902	1.385	2.125	3.259	5.000
Glycine-potassium acetate buffer of pH 7.2, in ml	4.750	4.617	4.412	4.098	3.615	1.876	1.741	—
Diluent: potassium salt solution M/15	14 ml into each tube							
Adsorbent: Bohemite from amalgamated aluminum, 20 mg Al <sub>2</sub> O <sub>3</sub> /ml	1 ml into each tube							
Final volume	20 ml in all tubes							
Number of Lf units per ml of final mixture	5	8	12	18	28	42	65	100
mg of Al <sub>2</sub> O <sub>3</sub> per ml of final mixture	1 mg into each tube							

**CONTROL:** A control was made using a series of adsorption tubes identical to the one listed in this table, with the exception of the adsorbent gel, which was substituted by water. After 24 hours, all tubes, control or not, were centrifuged at 1,500 r.p.m. during 10 minutes and the supernatant transferred to another tube. Each sediment was eluted in phosphate buffer of pH 7.2 in volumes such as to complete the original volume of 20 ml.

The three series of supernatants from the controls, tests and elutions were examined as for the number of Lf units, pH and macroscopical appearance. The number of Lf units in the control was taken as the number of Lf units added; the number of Lf units left in the supernatant, or the number eluted from the sediment, was measured directly; the number of Lf units adsorbed was calculated by difference.

The results of the experiments of the adsorption of toxoid dissolved in glycine-acetate buffer and diluted with monovalent chloride, divalent sulphate, trivalent phosphate or citrate and tetravalent ferrocyanide ions on Bohemite from amalgamated aluminum, Schmidt's gel and Bayerite are summarized in Tables IV, VI and VIII. Tables V, VII and VIII summarize the pH's of the



TABLE IV  
EFFECT OF THE CHARGE OF THE ANION ON THE ADSORPTION OF TOXOID BY BOEHMITE GEL



TABLE VI

ADSORPTION OF TOXOID BY SCHMIDT'S  
ALUMINUM HYDROXIDE GEL

DILUENT	TUBE	I	II	III	IV
M/15	Lf/ml before adsorption	8	18	45	105
KC <sub>1</sub> solution	Lf/ml adsorbed	8	18	45	40
	Lf/ml eluted	6	15	35	30
M/15	Lf/ml before adsorption	8	18	45	100
K <sub>2</sub> SO <sub>4</sub> solution	Lf/ml adsorbed	8	18	45	35
	Lf/ml eluted	2	15	35	25
M/15	Lf/ml before adsorption	8	188	45	100
K <sub>3</sub> PO <sub>4</sub> solution	Lf/ml adsorbed	0	0	0	0
	Lf/ml eluted	0	0	0	0
M/15	Lf/ml before adsorption	8	18	45	100
K <sub>4</sub> Fe(CN) <sub>2</sub> solution	Lf/ml adsorbed	8	14	18	20
	Lf/ml eluted	6	12	17	17
Adsorbent: Schmidt's aluminum hydroxide gel — 1 mg/ml					

TABLE VII

PH OF THE EXPERIMENTS REPORTED IN  
TABLE VI

DILUENT	TUBE	I	II	III	IV
M/15	pH before adsorption	6.5	6.7	6.7	6.7
KC <sub>1</sub> solution	pH after adsorption	6.4	6.4	6.5	6.6
	pH of the eluate	7.4	7.4	7.4	7.4
M/15	pH before adsorption	6.4	6.4	6.4	6.6
K <sub>2</sub> SO <sub>4</sub> solution	pH after adsorption	6.8	6.7	6.7	6.8
	pH of the eluate	7.4	7.4	7.4	7.4
M/15	pH before adsorption	11.3	11.3	11.3	11.3
K <sub>3</sub> PO <sub>4</sub> solution	pH after adsorption	11.1	11.1	11.1	10.9
	pH of the eluate	7.7	7.7	7.4	7.4
M/15	pH before adsorption	6.9	7.4	7.2	7.7
K <sub>4</sub> Fe(CN) <sub>2</sub> solution	pH after adsorption	7.3	7.3	7.2	7.2
	pH of the eluate	7.2	7.2	7.2	7.3

TABLE VIII

ADSORPTION OF TOXOID BY BAYERITE GEL

DILUENT	TUBE	I	pH of the solution
M/15 solution	Lf/ml before adsorption	115	7.1
	Lf/ml eluted	0	7.2
M/15 K <sub>2</sub> SO <sub>4</sub> solution	Lf/ml before adsorption	115	6.9
	Lf/ml after adsorption	0	7.1
	Lf/ml eluted	0	7.2
M/15 K <sub>3</sub> PO <sub>4</sub> solution	Lf/ml before adsorption	115	7.5
	Lf/ml after adsorption	0	7.6
	Lf/ml eluted	0	7.2
M/15 K <sub>4</sub> Fe(CN) <sub>2</sub> solution	Lf/ml before adsorption	110	7.7
	Lf/ml after adsorption	0	7.7
	Lf/ml eluted	0	7.2
Adsorbent: Bayerite gel — 1 mg/ml			



From these tables it is evident that the adsorptive power of the gels is the same in presence of sulphate and chloride ions, but is smaller in presence of ferrocyanide or citrate and zero in presence of phosphate ion. This result, of equal amounts of adsorption of toxoid in presence of chloride and sulphate ions, is important with regard to the preparation of Schmidt's aluminum hydroxide gel for foot-and-mouth disease vaccine because, if there is a direct correlation between the amounts of adsorption of diphtheria toxoid and foot-and-mouth disease virus (8), it is not necessary to eliminate the sulphate ion very carefully, which is a troublesome step in the preparation of this gel. From these results it can be concluded that the influence of phosphate ion on the adsorption is not merely due to the value of its charge or valency, since the amount of adsorption in presence of phosphate is not intermediary between chloride, sulphate, citrate and ferrocyanide and does not depend on the di or trivalency of the phosphate anion.

Hence, a second working hypothesis has to be suggested for the mechanism of the nature of the inhibiting action of phosphate ion: the action of the phosphate ion would consist in the blocking of some adsorption sites on the surface of the aluminum oxide hydrate particles, instead of a mere adsorption of the polyvalent ion in the diffuse double layer, as it was first assumed. This hypothesis is in agreement with the fact shown in the experiments recorded in Table IV, that the adsorption isotherm of the toxoid in presence of chloride, sulphate, citrate and ferrocyanide ions is a chemisorption isotherm with a maximum, as found before (7). This hypothesis is also in agreement with the fact that with other aluminum hydroxide gels of different crystalline structure besides Boehmite, no adsorption occurs in presence of phosphate ion, as shown in the tables, because in all aluminum hydroxides the surface of the particles has the same nature, being only aluminum and hydrogen ions (18, 19).

From the above described experiments, no idea could be suggested as for the nature of the adsorption sites, however, in the model given by Weiser (18) of the nature of the surface of an aluminum hydroxide particle in sol or gel states, this surface is covered mainly by completely or partly dissociated aluminum ions from the dissociation of  $\text{Al}(\text{OH})_3$  or  $\text{AlOOH}$  and by some adsorbed hydrogen ions. Hence, the more probable adsorption sites would be the aluminum ions on the surface; these aluminum ions would be linked in some way to the phosphate ion, most probably by exchange of hydroxyl ions, since the pH of the medium is neutral or slightly alkaline, with formation of an insoluble compound thus blocking the aluminum ions. This assumption is reasonable, taking in consideration the following experimental facts: a) aluminum phosphate is a highly insoluble substance, whereas aluminum ferrocyanide is less soluble than phosphate; b) soluble phosphate ion is strongly adsorbed by aluminum hydroxide gels, displacing other adsorbed anions (20); c) soluble



phosphate ion reacts with solid Gibbsite and with the Kaolin minerals, giving the dihydroxy-aluminum dihydrogen-phosphate — variscite — of the formula  $\text{Al}(\text{OH})_2 \cdot \text{H}_2\text{PO}_4$  (21, 22); the formation of this insoluble aluminum phosphate is the main mechanism for fixation of phosphate ion in soils (23).

The possibility of formation of an insoluble aluminum-toxoid compound in the precipitation of toxoid by alum was also suggested by Holt (12). The suggested mechanism for the action of phosphate is not in disagreement with Holt's experiments (12), in which he found that aluminum phosphate — ( $\text{AlPO}_4$ ) — gels adsorb appreciable amounts of toxoid, because he also observed that: a) phosphate ions inhibit the adsorption of toxoid either on aluminum hydroxide or phosphate gels; b) washed aluminum phosphate gel has a greater adsorptive power for toxoid than unwashed gel. Holt's observations would be in agreement with the mechanism suggested above, with the further assumption that there are free aluminum ions on the surface of the washed aluminum phosphate particles and that these ions are blocked in presence of phosphate ion by the formation of variscite as in aluminum hydroxide particles. From these arguments, it may also be concluded that the toxoid is probably linked to the hydroxide or phosphate gels by means of its carboxyl groups, as the hydrogen binding of carboxyl groups of polymers to hydroxyl groups of cellulose (24).

TABLE IX

EFFECT OF ANIONS ON THE ADSORPTION BY ALUMINUM HYDROXIDE GEL FROM  $\text{AlCl}_3 + \text{NH}_4\text{OH}$  PRECIPITATED "IN SITU"[illegible]



Based on this conclusion we decided to measure the adsorptive power for toxoid of a freshly precipitated amorphous aluminum hydroxide gel (25) or an aluminum hydroxide gel precipitated "in situ" in presence of toxoid, because, due to the larger number of aluminum ions on the surface of the particles, they presumably have a greater adsorptive power for diphtheria toxoid than the crystallized hydroxides listed in Table II; it was also investigated if phosphate ion prevents the adsorption in these hydroxides.

*Adsorption of Toxoid by Aluminum Hydroxides Precipitated "in situ".* — Solutions of aluminum chloride and ammonium hydroxide were used as reagents of formation of the aluminum hydroxide gel; toxoid, dissolved in M/15 glycine-acetate of pH 7.2, was used in all experiments. Table IX shows the results of the preliminary experiments in presence of 0.85% sodium chloride, M/15 glycine-acetate buffer of pH 7.2, phosphate buffer of pH 7.2, and 2.34% sodium sulphate solutions as diluents.

The toxoid was distributed in increasing amounts, in a series of 8 tubes of 3.5x9 mm marked outside with a line indicating the level corresponding to 20 ml of content. In general, the distribution was made with a constant increase from 5 until 100 Lf units per ml of final mixture. The volume of each tube was raised to the volume of toxoid of the last tube by addition of glycine-potassium acetate buffer of pH 7.2. Following the last addition of reagent, the necessary amount of diluent was added to complete a final volume of 20 ml. A 10%  $\text{NH}_4\text{OH}$  and a 16%  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$  solution (w/v) were added in amounts corresponding to the desired number of milligrams of  $\text{Al}_2\text{O}_3$  per ml for a final volume of 20 ml. After addition of the last reagent, the tubes were shaken by hand and then left standing at room temperature for 24 hours.

It is clearly shown in Table IX that phosphate ion inhibits completely the adsorption of diphtheria toxoid, whereas chloride, sulphate or glycine-acetate buffer do not. Furthermore, it was observed that aluminum hydroxide gel precipitated "in situ" has a very high adsorptive power, being about one thousand times greater than the other aluminum oxide gels used in former experiments (7). It was also observed that the adsorption follows a chemisorption isotherm. An experiment was performed using smaller amounts of aluminum hydroxide gel in order to verify its maximum adsorptive power for diphtheria toxoid: Table X shows the result thus obtained.

The maximum adsorptive power found was of 800 Lf units per milligram of  $\text{Al}_2\text{O}_3$  of amorphous aluminum hydroxide gel, and the constant saturation volume (7) was about 500 Lf's per mg of  $\text{Al}_2\text{O}_3$ . These results are in agreement with Holt's observations that very small amounts of aluminum as aluminum hydroxide can precipitate large amounts of toxoid, probably in form of an insoluble aluminum-toxoid compound (12).



TABLE X

DETERMINATION OF THE MAXIMUM ADSORPTION OF TOXOID BY ALUMINUM HYDROXIDE  
GEL PRECIPITATED "IN SITU"

TUBE	I	II	III	IV	V	VI	VII	VIII	IX	*X
Lf/ml added	5	8	12	20	33	55	83	135	220	350
Lf/ml adsorbed	5	8	12	20	33	55	80	60	53	50
pH before adsorption	7.0	7.0	7.0	7.0	6.9	6.9	6.9	6.9	6.9	6.8
pH after adsorption	6.3	6.2	6.6	6.2	6.4	5.9	6.65	6.9	7.0	7.0

Amorphous aluminum hydroxide gel — 0.1 mg  $\text{Al}_2\text{O}_3$ /ml  
Diluent — 0.85% NaCl to complete 20 ml of final mixture.

Experiments were conducted to verify if the ionic strength of medium containing NaCl influences the adsorption: the results are presented in Table XI. The amorphous aluminum hydroxide, however, showed in the experiments a higher adsorptive power, ranging from 980 to 1000 Lf units per mg of  $\text{Al}_2\text{O}_3$ .

TABLE XI

EFFECT OF CONCENTRATION OF NaCl SOLUTION USED AS DILUENT ON THE ADSORPTION OF  
TOXOID BY ALUMINUM HYDROXIDE GEL PRECIPITATED "IN SITU"

TUBE	I	II	III	IV	V	VI	VII	VIII
ml of toxoid in glycine-acetate	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3
ml solution acetate-glycine	5.7	5.7	5.7	5.7	5.7	5.7	5.7	5.7
10.8 ml of a solution of NaCl of the following molarity	2M	1M	M/2	M/4	M/8	M/16	M/32	0
Lf/ml added	98	98	98	95	100	100	100	98
Lf/ml adsorbed	98	98	98	95	100	100	98	98
pH before adsorption	6.8	6.9	7.0	7.0	7.1	7.1	7.1	7.2
ph after adsorption	6.0	6.0	6.1	6.2	6.2	6.2	6.2	6.2

Amorphous aluminum hydroxide gel — 0.1 mg of  $\text{Al}_2\text{O}_3$ /ml

No significant variation in the adsorptive power in relation to the ionic strength was found.

Also, no significant difference was found in further experiments in the adsorptive power of hydroxide precipitated "in situ" in the pH range of 4.6 to 7.0: in these experiments, an adsorptive power of 900 Lf units per mg of  $\text{Al}_2\text{O}_3$  as amorphous aluminum hydroxide was measured.



*Influence of the Anion Charge on the Adsorptive Power of Amorphous Aluminum Hydroxide Precipitated "in situ".* — Since, from the view point of amount of adsorption of toxoid, this gel showed to be the most interesting of all hydroxides used, we decided to investigate also the influence of the charge or valency of the anions present in the medium upon the adsorption of diphtheria toxoid. The following M/15 potassium salt solutions were used as the medium in which the aluminum chloride was precipitated, either by ammonium or potassium hydroxide: potassium chloride, potassium fluoride, potassium nitrate, potassium pyrophosphate and potassium citrate; the experiments were performed with both ammonium and potassium chloride, because Holt (12) found that ammonium alum is a better precipitant of toxoid than potassium alum. Toxoid dissolved in M/15 glycine-acetate buffer of pH 7.2 was used in all experiments. The amounts of reagents were the same as those used in the experiments of Table X. Table XII presents the results of the experiments on the influence of the charge of anions of different potassium salts on the adsorption of toxoid by aluminum hydroxide gel from  $\text{AlCl}_3$  solutions, either by  $\text{NH}_4\text{OH}$  or  $\text{KOH}$  solutions.

These results show that the precipitate from  $\text{NH}_4\text{OH}$  has a greater adsorp-

TABLE XII

INFLUENCE OF THE CHARGE OF THE ANION OF POTASSIUM SALTS ON THE ADSORPTION OF TOXOID BY AMORPHOUS ALUMINUM HYDROXIDE GEL PRECIPITATED "IN SITU" FROM  $\text{AlCl}_3$  BY  $\text{NH}_4\text{OH}$  OR  $\text{KOH}$

Diluent: M/15 solution of	precipitation by $\text{NH}_4\text{OH}$		precipitation by $\text{KOH}$	
	number of Lf units adsorbed by mg $\text{Al}_2\text{O}_3$	pH after adsorption	number of Lf units adsorbed by mg $\text{Al}_2\text{O}_3$	pH after adsorption
potassium chloride	greater than 870 Lf	6.3-7.0	greater than 40 Lf	4.4
potassium nitrate	greater than 950 Lf	6.2	—	—
potassium fluoride	6.5-172 Lf + 50 precipitate not of $\text{Al}(\text{OH})_3$	5.1-8.6	greater than 25 Lf — the precipitate is not $\text{Al}(\text{OH})_3$	4.8-7.5
potassium sulphate	greater than 900 Lf	6.3-7.0	greater than 38 Lf	6.8
potassium oxalate	no precipitation of $\text{Al}(\text{OH})_3$	6.8	—	—
potassium triphosphate	—	—	no adsorption	10.4
potassium ferrieyanide	greater than 900 Lf	6.0	—	—
potassium citrate	—	—	no precipitation of $\text{Al}(\text{OH})_3$	9.0
sodium citrate	no precipitation of $\text{Al}(\text{OH})_3$	7.5	no precipitation of $\text{Al}(\text{OH})_3$	—
potassium ferrocyanide	450 Lf	7.9	100 Lf	7.2
potassium pyrophosphate	no adsorption	9.3	no adsorption	—
potassium sodium tartrate	no precipitation of $\text{Al}(\text{OH})_3$	6.8	no precipitation of $\text{Al}(\text{OH})_3$	—
sodium tetraborate	no adsorption — precipitate not $\text{Al}(\text{OH})_3$	4.5-8.4	no adsorption — precipitate not $\text{Al}(\text{OH})_3$	4.0-8.9



tive power than the precipitates from KOH, in agreement with Holt's findings; however, the nature of this effect is not yet understood. In most cases no precipitation of  $\text{Al}(\text{OH})_3$  was obtained, either due to complex formation or to formation of precipitates having composition different from  $\text{Al}(\text{OH})_3$ . These results also confirm the previous observations that the inhibiting effect of phosphate ion on the adsorption of toxoid by aluminum hydroxide gels is not due to the charge or valency of the ion, but to a specific effect of the phosphate on the surface of the aluminum hydroxide particles.

#### SUMMARY

1.<sup>o</sup>) Phosphate ion has a strong inhibiting power on the adsorption of diphtheria toxoid by aluminum hydroxide gels of different crystalline structures and surface areas.

2.<sup>o</sup>) The probable mechanism of inhibition is the blocking of aluminum ions from the  $\text{AlOOH}$  or  $\text{Al}(\text{OH})_3$  molecules on the surface of the colloidal particles due to the formation of an insoluble aluminum phosphate, probably variscite —  $\text{Al}(\text{OH})_2 \cdot \text{H}_2\text{PO}_4$ .

3.<sup>o</sup>) Chloride, glycine, acetate and sulphate ions do not have a noticeable inhibiting effect on the adsorption of toxoid by aluminum hydroxide gels; ferrocyanide ion has a small inhibiting power, but in smaller degree than phosphate ion. In media containing these ions, the adsorption of toxoid follows roughly a chemisorption isotherm with a maximum value for the adsorptive power. This adsorptive power of the different aluminum hydroxides is directly proportional to the surface area of the gel or inversely proportional to the average particle size; no direct relation was found between the crystalline structure and adsorptive power of the different aluminum hydroxide gels.

4.<sup>o</sup>) Adsorptive powers for toxoid ranging between 800 and 1000 Lf units per milligram of  $\text{Al}_2\text{O}_3$  were found with amorphous aluminum hydroxide precipitated "in situ" from diphtheria toxoid dissolved in glycine-acetate buffer of pH 7.1, using aluminum chloride and ammonium hydroxide solutions as precipitants; potassium hydroxide solutions give amorphous aluminum hydroxide gels with smaller adsorptive power than precipitates from ammonium hydroxide solutions; this adsorption can be completely inhibited by phosphate ions; aluminum complexing ions such as tartrate, citrate and oxalate prevent precipitation of aluminum hydroxide and no toxoid is adsorbed.

#### RESUMO

1.<sup>o</sup>) O ion fosfato tem um forte poder inibidor em relação à adsorção de toxóide diftérico por géis de hidróxido de alumínio de diferentes estruturas cristalinas e áreas específicas.



2.º) O mecanismo provável desse poder inibidor é o bloqueio dos ions alumínio das moléculas  $\text{AlOOH}$  ou  $(\text{Al}(\text{OH})_3)$  da superfície das partículas coloidais devido à formação de um fosfato de alumínio insolúvel, que é provavelmente variscita —  $\text{Al}(\text{OH})_2\text{H}_2\text{PO}_4$ .

3.º) Os ions clorêto, glicina, acetato e sulfato não apresentam um efeito inibidor apreciável na adsorção de toxóide por géis de hidróxidos de alumínio; o ions ferrocianêto tem um pequeno poder inibidor, porém em grau inferior ao do ion fosfato. Em meios contendo êsses ions, a adsorção do toxóide segue aproximadamente uma isoterma de adsorção química, com um valor máximo para o poder de adsorção. Êsse poder de adsorção é diretamente proporcional à dimensão média das partículas; não foi evidenciada nenhuma correlação direta entre o poder de adsorção e a estrutura cristalina dos diferentes géis de hidróxidos de alumínio.

4.º) Foram observados poderes de adsorção para o toxóide ocorrendo entre 800 e 1000 unidades Lf por miligrama de  $\text{Al}_2\text{O}_3$ , no caso de hidróxido de alumínio amorfo precipitado "in situ" em uma solução de toxóide diftérico dissolvido em tampão glicina, acetato de pH 7,1, sendo usados como agentes precipitantes soluções de clorêto de alumínio e de hidróxido de amônio; soluções de hidróxido de potássio produzem géis de hidróxido e alumínio amorfo tendo poder de adsorção menor do que os precipitados obtidos com soluções de hidróxido de amônio; esta adsorção pode ser completamente inibida por ions fosfato; ions complexantes do alumínio, como os ions, citrato e oxalato, impedem a precipitação do hidróxido de alumínio e nenhum toxóide é absorvido.

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